THE ROYAL SOCIETY

Published online 27 June 2002

Structure and function of antifreeze proteins

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High-resolution three-dimensional structures are now available for four of seven non-homologous fish and insect antifreeze proteins (AFPs). For each of these structures, the ice-binding site of the AFP has been defined by site-directed mutagenesis, and ice etching has indicated that the ice surface is bound by the AFP. A comparison of these extremely diverse ice-binding proteins shows that they have the following attributes in common. The binding sites are relatively flat and engage a substantial proportion of the protein's surface area in ice binding. They are also somewhat hydrophobic—more so than that portion of the protein exposed to the solvent. Surface–surface complementarity appears to be the key to tight binding in which the contribution of hydrogen bonding seems to be secondary to van der Waals contacts.

Keywords: antifreeze proteins; α-helix; ice binding; surface complementarity; thermal hysteresis; van der Waals interactions

1. INTRODUCTION TO AFPS AND THEIR PROTECTIVE EFFECTS ON FISHES

AFPs can be defined as proteins that have an affinity for ice. In a situation where an ice front is in equilibrium with an aqueous solution, a slight undercooling of the solution below the equilibrium freezing point will lead to water molecules joining the ice lattice. As a result, the ice front will advance. Most solutes, including the vast majority of proteins (e.g. bovine serum albumin and myoglobin) will be excluded and pushed ahead of the expanding ice front. AFPs are different in that they adsorb to the ice (Raymond & DeVries 1977). By doing so, they restrict the growth of the ice front to regions between the adsorbed protein molecules. These regions grow with a local curvature that makes it thermodynamically unfavourable for water molecules to add to the ice lattice (Wilson 1993). This results in non-colligative, non-equilibrium lowering of the freezing point. In the presence of AFPs, ice crystals remain the same size for hours or days at temperatures between the colligative freezing point (melting point) and the lower non-equilibrium freezing point (figure 1). The difference between these two temperatures is referred to as thermal hysteresis and is a function of the AFP concentration.

The protective effect that thermal hysteresis has on an organism is perhaps easiest to illustrate for marine teleosts. AFPs in the blood and peripheral tissues can depress their freezing point below that of the surrounding seawater (DeVries 1983). Just over 1 °C of freezing point depression (thermal hysteresis) is sufficient to protect the fishes from freezing in icy seawater, which can be as cold as −1.9 °C. This is because thermal hysteresis acts additively with the colligative lowering of the freezing point due to serum solutes. The latter accounts for 0.7–0.9 °C of the freezing point depression.

2. RECENT EVOLUTION OF AFPS MAY ACCOUNT FOR THEIR DIVERSITY

In the 30 years following the discovery of antifreeze glycoproteins in Antarctic Notothenioids by Art DeVries and his colleagues (DeVries & Wohlschlag 1969; DeVries *et al.* 1970), several other AFP types have been characterized in distinct groups of teleosts in both the northern and southern hemispheres (Fletcher *et al.* 2001). These discoveries came principally from the laboratories of Art DeVries in Illinois and of Choy Hew and Garth Fletcher in Newfoundland. The AFP types are radically different in their primary sequences and 3D structures and yet they all bind to ice and depress the non-equilibrium freezing point below the melting point. Moreover, the distribution of these different types does not seem to fit with the evolutionary relationships of the host fishes. For example, type II AFP, which is a homologue of the carbohydrate-recognition domain of Ca^{2+}-dependent lectins, is found in three very distantly related fishes (smelt, herring and sea raven) that belong to different super orders (Fletcher *et al.* 2001). By contrast, three very closely related fishes (shorthorn sculpin, longhorn sculpin and sea raven) that are in the same genus or family produce completely unrelated AFP types. In the mid-1980s, when this diversity was first uncovered, Gary Scott, a postdoctoral fellow in our laboratory, correlated the radiation of the bony fishes with the history of the Earth's climate (Scott *et al.* 1986). We thus became aware that sea-level glaciation was a recent phenomenon; when the teleosts began their evolution and expansion 175 Myr ago, the oceans were uniformly warmer and did not have any build-up of ice. As a result, it was only after the present sub-orders, families and genera were established that fishes were challenged

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One contribution of 15 to a Discussion Meeting Issue 'Coping with the cold: the molecular and structural biology of cold stress survivors'.

Figure 1. Ice crystal stasis in the presence of winter flounder AFP. An ice crystal formed within a glass capillary containing winter flounder AFP stock solution takes this typical hexagonal bipyramidal shape due to AFP binding to the {20–21} pyramidal planes of ice (Knight *et al.* 1991). It can be maintained without growth or shrinkage at temperatures between the melting point and the non-equilibrium freezing point of the AFP solution, i.e. within the thermal hysteresis gap.

with the threat of freezing. The remarkable diversity of AFP types in fishes shows that a number of dissimilar proteins have adapted to the task of binding ice. This is atypical of protein evolution. Most proteins that serve the same function in different organisms do so as a result of direct descent from an ancestral form. For example, citrate synthase, an enzyme in the tricarboxylic acid cycle, is essentially the same protein in all aerobes, having been required throughout their evolution.

The need for AFPs in marine fishes is very recent, explaining the structural diversity in fish AFPs. However, there are indications that AFPs may be equally diverse in other phyla. Although only two insect AFP types have been structurally characterized, they are non-homologous. Plant AFPs too, show considerable diversity (Hon *et al.* 1995; Worrall *et al.* 1998; Sidebottom *et al.* 2000). One could speculate again that climate change impinged on these phyla after their evolutionary divergence, but another reason why AFP diversity may arise and be maintained is that ice can present many different surfaces with different geometric arrangements of oxygen atoms (figure 2). Any protein with complementarity for one of these planes might serve as an antifreeze prototype on which natural selection can act to improve binding efficiency. In the case of the type II AFP-producing fishes, it is probable that C-type lectins have been coopted at different times in the evolution of teleosts and fashioned into antifreezes (Fletcher *et al.* 2001), whereas in Cottid fish, different proteins have served as precursors, at least two of which bind to different planes of ice.

3. LIMITATIONS OF THE HYDROGEN-BONDING HYPOTHESIS FOR AFP BINDING TO ICE

Type I AFP found in flounders and sculpins is a small alanine-rich, amphipathic, α-helix. It is the simplest of the fish AFPs and the one that has been most extensively used as a model system for trying to understand how these proteins bind to ice. When DeVries & Lin (1977) first sequenced one of the type I AFP isoforms from winter flounder, the repeating structure of this 37 amino acid peptide suggested a way in which it might bind to ice. In the model proposed by DeVries, the threonine and aspartic acid residues, each of which occur with an 11 amino acid repeat, form hydrogen bonds to oxygen atoms on the primary prism plane of ice (DeVries 1984). In particular, the proposed distance of 4.5 Å between the threonine hydroxyl and the carboxyl group of the aspartates was matched to the 4.5 Å spacing between the oxygen atoms on this plane, an arrangement that was repeated three times along the helix (figure 3*a*). This was the birth of the hydrogen-bonding hypothesis, which has since dominated the discussion of antifreeze mechanisms. Subsequently, the binding plane for winter flounder type I AFP was determined by ice etching and shown to be the {20–21} pyramidal plane (Knight *et al.* 1991). This did not pose any serious problems for the hydrogen-bonding hypothesis because it was possible to model the AFP to this new plane, as previously shown by others (Chou 1992; Wen & Laursen 1992*a*). The latter authors matched the 16.5 Å repeats of Thr and Asx to repeating surface structure on this plane of ice (figure 3*b*). Concerns about the low number and weakness of hydrogen bonds for binding an AFP to ice were partly allayed by the suggestion of Knight *et al.* (1993) that hydrogen-bonding groups like the threonine hydroxyl might occupy oxygen atoms in the ice lattice. In this way, they could form additional hydrogen bonds and effectively freeze the antifreeze into the top layer of the ice lattice (figure 3*c*). Many variations of these models appeared in the 1990s to account for the hydrogen bonding of AFP to ice (e.g. Jorgensen *et al.* 1993; Lal *et al.* 1993; McDonald *et al.* 1993; Madura *et al.* 1994; Sicheri & Yang 1995; Cheng & Merz 1997). However, to paraphrase the motto of The Royal Society '*Nullius in verba*', you can only go so far with molecular models before you have to test them by experimentation.

4. DEFINITION OF THE ICE-BINDING SITE OF TYPE I AFP BY EXPERIMENTATION

One of the advantages of working with type I AFP is that it is small enough to be made by solid-phase peptide synthesis (Chakrabartty *et al.* 1989), allowing structure–function studies to progress (Wen & Laursen 1992*b*). In the mid-1990s it became clear in experiments where some or all of the regularly spaced threonines were replaced with serines and valines, that hydrogen bonds may not be the key to ice binding (Chao *et al.* 1997; Haymet *et al.* 1998; Zhang & Laursen 1998). Thus, when the middle two threonines (T12 and T23) of a winter flounder AFP were replaced by serines, almost all of the activity was lost, even though serine can potentially form hydrogen bonds just as effectively as threonine. By contrast, replacement by valine caused little loss of activity, indicating that the methyl group of threonine might be important for ice binding (Chao *et al.* 1997). Subsequently, we compared the sequences of five type I isoforms from three different right-eye flounders and came to the realization that the hydrophilic surface of the helix is very variable. On the contrary, the opposite, hydrophobic face consisting of regularly spaced alanines and threonine was highly conserved

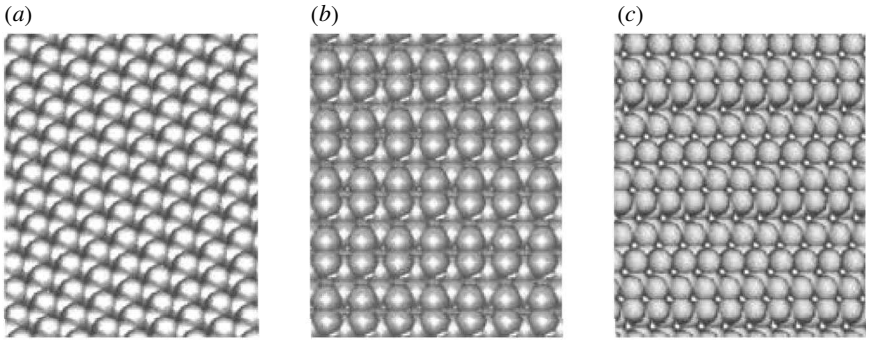


Figure 2. Atomic topographies of three ice planes: (*a*) basal, (*b*) primary prism and (*c*) a {20–21} pyramidal plane shown at atomic resolution to illustrate their different surface contours. The ice section models were generated using SYBYL.

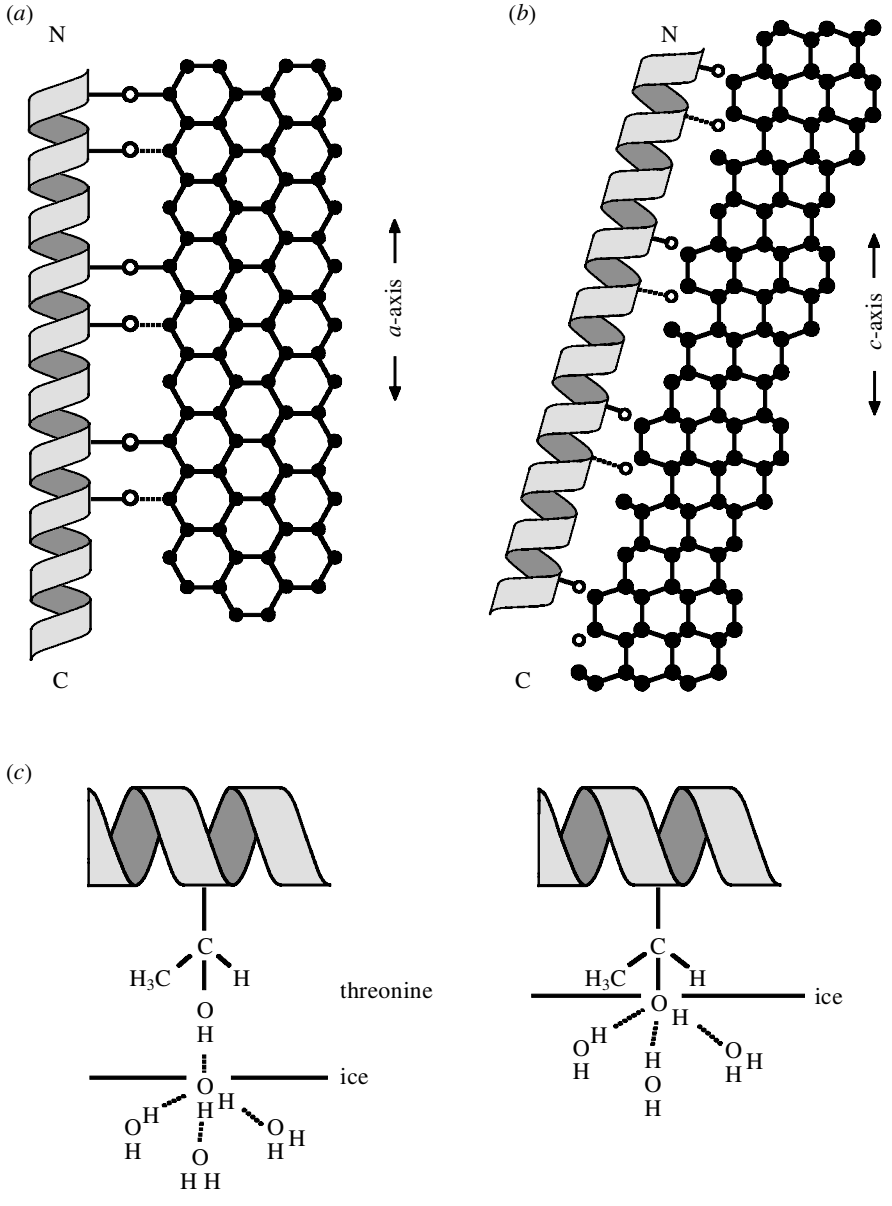
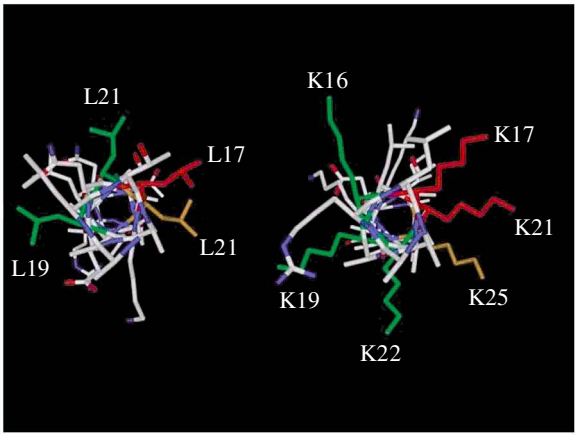


Figure 3. Illustrations of the hydrogen-bonding hypothesis for AFP binding to ice. (*a*) A representation of the original model of DeVries (1983) showing winter flounder (type I) AFP hydrogen bonding through its Thr and Asx side chains to oxygen atoms on the primary prism plane of ice. Dotted lines from the Asx residues indicate hydrogen bonding to an oxygen atom one rank behind the one illustrated. (*b*) A revision of the hydrogen-bonding hypothesis proposed by Wen & Laursen (1992*a*) to accommodate the discovery of the ice plane bound by winter flounder AFP (Knight *et al.* 1991). Thr and Asx side chains are within hydrogen-bonding range of oxygen atoms on the {20–21} plane of ice in the ⟨01–12⟩ direction. (*c*) A general modification of the hypothesis by Knight *et al.* (1993) illustrates how additional hydrogen bonds can be formed if a functional group such as a Thr OH group occupies the top layer of ice.

(*a*)



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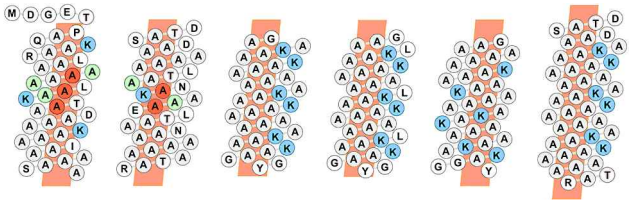

Figure 4. (*a*) End-on projections of type I AFPs showing Leu or Lys substitutions at points around the helix. Substitutions into winter flounder HPLC-6 (left) and shorthorn sculpin sculpin SS-8 (right) that block ice binding are coloured red. Those that allow binding but decrease antifreeze activity are coloured orange and those that have no effect on activity are coloured green. (*b*) Helical net projections of type I AFPs. One structural requirement for antifreeze activity in the single α-helix AFPs (type I) appears to be an uninterrupted section of alanines running the length of the helix. This is designated as the ice-binding site and is indicated by the orange background. Alanines where steric substitutions eliminate antifreeze activity in AFPs from shorthorn sculpin (SS-8) and winter flounder (HPLC-6) are coloured red. Those that are compatible with activity are coloured green. Basic residues are shown in blue and threonines in pink.

(Baardsnes *et al.* 1999). If the most important function of the helical AFP is to bind to ice, then the ice-binding face should be highly conserved. Only the alanine-rich surface appears to meet this criterion. This indicated that the alanine-rich surface might indeed be the ice-binding face of the helix. We set out to test this hypothesis by substituting alanine residues with leucine at points around the helix. The rationale for this substitution was that a longer side chain might sterically prevent the AFP from contacting ice. To this end, we made four individual substitutions, A17L, A19L, A20L and A21L, and tested them for thermal hysteresis activity (figure 4*a*, left). A19L and A20L, where the leucine side chains project from the hydrophilic surface, had little effect on activity and produced a wild-type ice crystal. A21L, however, had very low activity. It shaped the ice crystal into a hexagonal bipyramid but was unable to prevent it from growing. A17L was completely inactive and failed to shape the ice at all. Based on these results, we suggested that the ice-binding surface, which had never been experimentally determined, was in fact the alanine-rich surface. Although it did encompass the regularly spaced threonine residues, it did not extend to the neighbouring asparagines and it was the methyl group of the threonines that was more important for binding than the hydroxyl.

The antifreeze field has not readily accepted this about-face regarding the ice-binding surface. Partly for this reason, we set out to determine the ice-binding surface of type I AFP from shorthorn sculpin. It is still not clear if the sculpin type I AFP is a homologue of the flounder antifreeze or has arrived at a similar amphipathic α-helical structure by convergent evolution. If it is a homologue, these peptides may well have been coopted as antifreezes on different occasions in the same way that C-type lectins became type II AFPs. The ice surface bound by the SS-8 isoform from shorthorn sculpin is the secondary prism plane {11–20} (Knight *et al.* 1991). The currently accepted ice-binding face of the sculpin type I AFP is the lysine- and arginine-rich surface along which there is some periodicity to the placement of these basic residues. The lysines on this hydrophilic surface have been docked to the secondary prism plane of ice (Wierzbicki *et al.* 1996). This model, where a different antifreeze sequence binds to a different plane of ice, has provided a second opportunity to test the hydrogen-bonding hypothesis. Again, we replaced alanines at points around the helix with a longer-chained amino acid. In this study, we used lysine rather than leucine to improve the solubility of the variant peptides (figure 4*a*, right). One other advantage of using lysine in this instance is that as it has been postulated to be an ice-binding residue, the position of the residue is being tested rather than the nature of the residue. The variants, A16K, A17K, A19K, A21K, A22K and A25K, provided a similar range of phenotypes to the leucine series in flounder AFP. On the hydrophilic surface, alanines 16, 19 and 22 could be replaced by lysine without penalty (Baardsnes *et al.* 2001). But on the hydrophobic surface, A17K and A21K completely eliminated antifreeze activity and ice shaping. A25K may be on the edge of the ice-binding surface in that it had no thermal hysteresis activity, but it did shape ice into a hexagonal bipyramid.

Having redefined the ice-binding site of SS-8, we have modelled the docking of this hydrophobic face to the secondary-prism plane of ice (Baardsnes & Davies 2001). The helix repeat, which is also 16.5 Å, places the alanine methyl side chains within a groove on this plane of ice such that there is an overall tight complementary fit. This presumably allows van der Waals and hydrophobic interactions to occur over the length of the helix. The redefinition of the sculpin AFP's ice-binding site has not been well received in some quarters. We have, therefore, reinterpreted data from the Laursen and Wierzbicki laboratories on copolymers of lysine and alanine and other type I-like AFP constructs (figure 4*b*). Zhang & Laursen (1999) had previously found that a repeating pattern of lysine on one side of the helix (LKAAK and AKAAK series) was compatible with activity, whereas a more distributive placement of lysine around the helix was incompatible with activity. Their interpretation was that the regular placement of lysines was important for binding to ice. Our interpretation is that the placement of lysines on

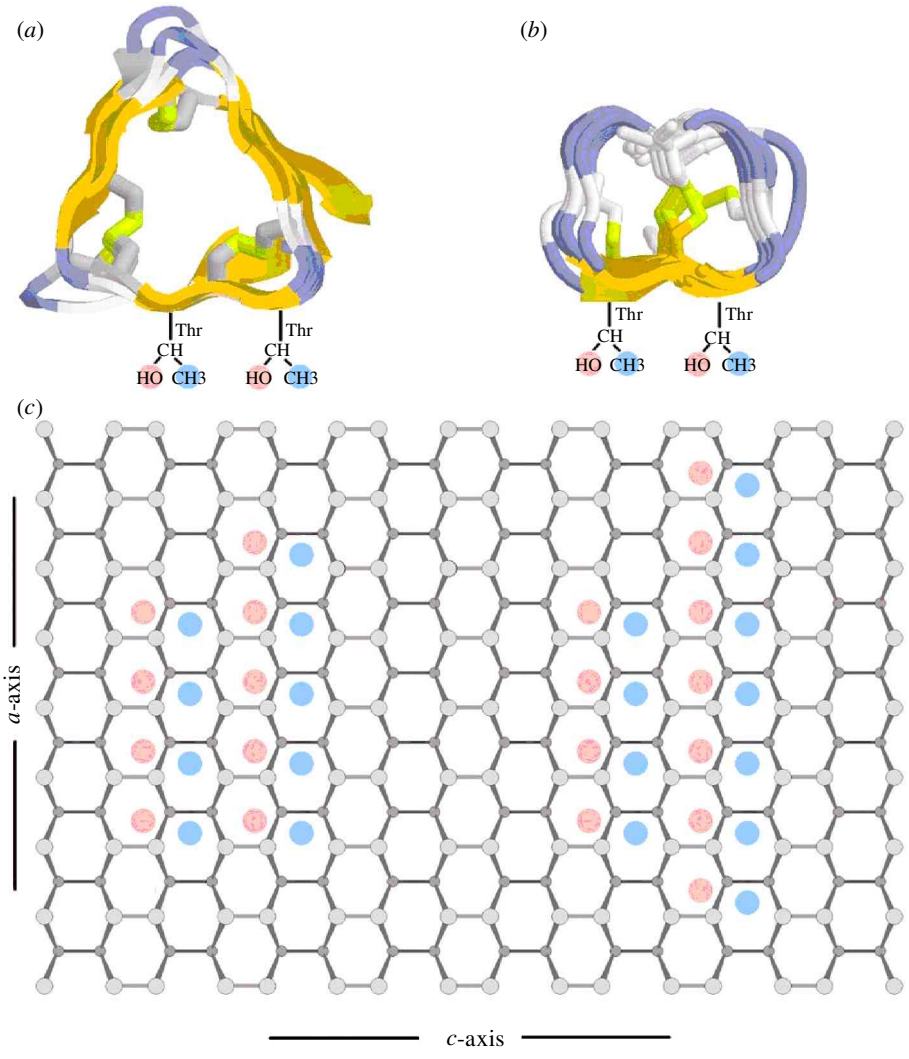

Figure 5. Structural match of moth and beetle AFPs to the primary prism plane of ice. The top section presents end-on projections of the β-helical AFPs from (*a*) spruce budworm, *Choristoneura fumiferana* and (*b*) the common yellow mealworm beetle, *Tenebrio molitor*. β-strands are shown in gold, turns in blue and disulphide bridges in yellow/green. Despite their radically different β-helical structure, the two insect AFPs present very similar arrays of Thr residues on one flat side of the AFP. (*c*) The remarkably good spatial match of the Thr methyl (blue dots) and hydroxyl (pink dots) groups to the primary prism plane of ice. In each case, the AFPs are rotated through 90 degrees from the upper view such that the top dots represent the Thr residues closest to the N terminus.

one face of the helix generates an uninterrupted alanine-rich surface adjacent to these long side chains. This we take to be the ice-binding site (figure 4*b*). The reason why poly-AK is inactive is because two lysines fall within the conserved alanine zone needed for ice binding. Similarly, the 43 mer synthesized by Wierzbicki *et al.* (2000) is active because it again has an unbroken tract of alanines on one face of the helix. We relegate the lysines to a role in promoting the solubility of what would otherwise be very hydrophobic peptides.

5. FISH AND INSECT AFPS: DIFFERENCES AND SIMILARITIES

We have extended structure–function studies to the newly characterized insect AFPs (Graether *et al.* 2000; Liou *et al.* 2000; Duman 2001) to see if there is some common theme for their ice-binding mechanism. It should be noted that insect antifreezes are considerably more active than those from fishes. At a concentration of 20 μM, AFP from the spruce budworm *Choristoneura fumiferana* (CfAFP) has roughly four times the thermal hysteresis activity of a 400 μM solution of type I AFP from winter flounder (Graether *et al.* 2000). We suspect that this difference in specific activity relates more to the ice crystal morphology than to the AFP's affinity for ice. CfAFP produces a hexagonal ice crystal that bursts along the *a*-axes, whereas all fish AFPs shape ice into a hexagonal bipyramid that typically bursts out of the tips along the *c*-axis. We suggest that the tips of the hexagonal bipyramid are the weak spots for containment of growth of the crystal. Thus, ice crystal shape may account for the lower specific activity of fish AFPs compared with insect AFPs. One explanation for the difference in ice crystal morphology is that we find CfAFP binds to the basal plane as well as the primary prism planes. This was demonstrated by ice etching studies (Graether *et al.* 2000). Recently, the X-ray crystal structure for CfAFP has been determined by Eeva Leinala in Zongchao Jia's laboratory (E. Leinala, unpublished results). The threonine arrays on

the ice-binding surface are even more regular than suggested by nuclear magnetic resonance and they look very similar to the modelling projections described by Graether *et al.* (2000). One outcome of the modelling was a demonstration of the striking match between the spacing of the threonine side chains and oxygen atoms on the primary prism (and basal) plane of ice. The other insect AFP that has been characterized at the level of its 3D structure comes from the beetle *Tenebrio molitor* (Liou *et al.* 2000). Although this AFP, like CfAFP, is also a β-helix, the two proteins are not homologous. Indeed, the beetle AFP is a right-handed helix with 12 or 13 amino acids per turn, in contrast to CfAFP that is a left-handed helix with 15 amino acids per turn (figure 5). Moreover, the two AFPs have completely different disulphide bonding patterns. Despite these structural differences, their ice-binding sites are virtually superimposable. The two ranks of threonine residues line up perfectly because the threonines are in the same rotameric configuration. This remarkable example of convergent evolution is illustrated in figure 5, where the alignment of methyl groups (red) and hydroxyl groups (blue) is shown against the backdrop of the primary prism plane of ice.

Elucidation of the 3D structure of TmAFP illustrated the possibility for the threonine hydroxyls to occupy oxygen atom positions in the top layer of the ice lattice (Liou *et al.* 2000). This was the model originally suggested by Knight *et al.* (1993) for the binding of type I AFP to ice that generated extra hydrogen bonds for the AFP–ice interaction. Following the realization that the threonine hydroxyls are not particularly important for type I AFP activity, it seemed unlikely that a lattice occupancy model could explain the tight binding of this antifreeze to ice. However, the model looked particularly persuasive in the case of TmAFP because the equivalent threonine-rich surface in CfAFP was demonstrated to be the ice-binding surface by site-directed mutagenesis (Graether *et al.* 2000). There were no steric clashes that would prevent the threonine side chains from occupying oxygen atoms in the surface layer. Moreover, there was a tightly bound water molecule between the threonines of each Thr-Xaa-Thr motif that also aligned with one of the surface oxygen atoms. Thus TmAFP could be docked into the top layer of the ice lattice both on the primary prism plane and the basal plane. Subsequently, it was realized that other docking positions were possible. One of these, where the threonine methyl group and the threonine hydroxyl both fit into small cavities on the primary prism plane, provides a particularly good surface–surface complementarity. In this way, the protein would sit snugly enough onto the ice to generate considerable van der Waals and hydrophobic interactions that could compensate for some loss of hydrogen bonds.

6. AFP-ICE CONTACT AND THE ANALOGY TO QUATERNARY STRUCTURE INTERACTIONS

In this regard, the binding of insect AFPs to ice is similar to that postulated for fish type I and type III AFP interactions with ice, and comparable with those seen between protein subunits in quaternary structure. This conclusion has the attraction that fish and insect AFPs use similar mechanisms for binding to ice. The key to the mechanism

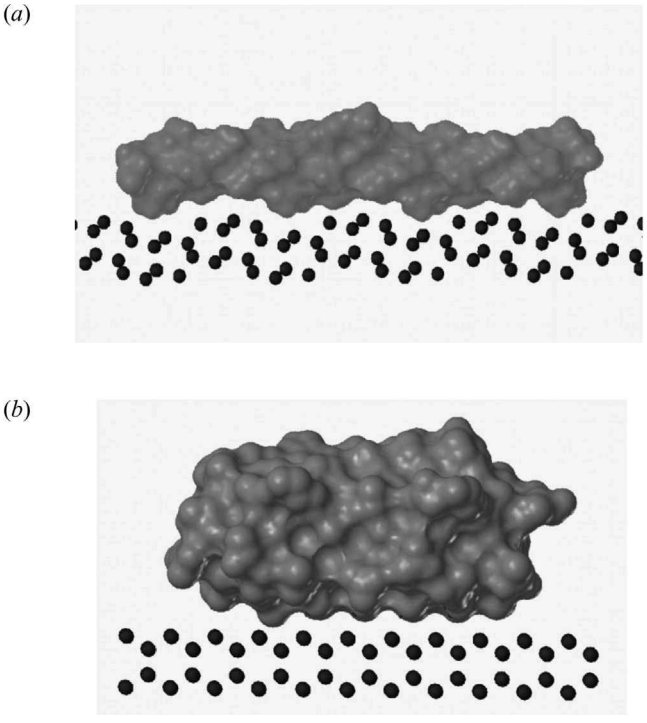

Figure 6. Potential for surface–surface complementarity between AFPs and ice. Lateral views of (*a*) winter flounder AFP and (*b*) *Tenebrio molitor* docked to pyramidal {20–21} and primary prism ice planes, respectively. The AFPs are shown in a space-filling form and, in the ice cross-section, dots represent oxygen atoms in the lattice.

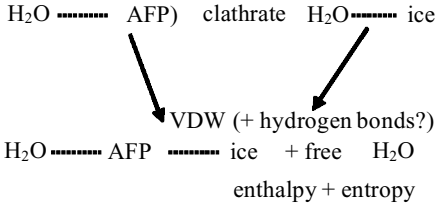

Figure 7. Contributions to the energetics of AFP binding to ice.

is that each AFP is shaped in such a way that a significant proportion of its surface area can dock to ice. The docking interaction is particularly intimate and is tailored for the specific ice-binding surface. For example, the type I AFP interactions with ice match the periodicity of the α-helix to the 16.5 Å undulation of the prism plane. Similarly, the insect AFPs with their 4.5 Å spacing between parallel β-strands fits extremely well into the 4.5 Å periodicity of both the primary prism and basal planes (figure 6). In terms of the energetics of binding, there are several components. These include enthalpic contributions from the van der Waals interactions that come from ideal surface–surface complementarity, together with adventitious hydrogen bonds that happen to be formed within the ice-binding site contact (figure 7). In addition, there may be an entropic component derived from not needing to solvate the rather hydrophobic ice-binding surface when it docks to ice (Sonnichsen *et al.* 1996).

P.L.D. and V.K.W. thank the numerous students, fellows and

collaborators who have contributed to this work. We are grateful to CIHR (formerly MRC), NSERC, PENCE and A/F Protein Inc. for financial support. P.L.D. is a Killam research fellow and J.B. was supported by an Ontario graduate scholarship.

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Discussion

D. Hall (*Multi-Disciplininary Research and Innovation Centre, North East Wales Institute, Wrexham, UK*). Do you have an estimate of the area of the binding surface of these

molecules onto ice? Roughly speaking, if you can quote it in square angstroms it would be easier for me.

P. L. Davies. A few hundred square angstroms.

D. Hall. Second question if I may. As far as insect AFPs are concerned, do they do the same job as the fish AFPs? In other words, when you talk about a fish AFP, the fish is in water which has ice crystals in it, and if it imbibes them through the gills then because of the fish's body fluid colligative properties versus the salinity of the water, the crystals must not grow, so that you can see quite reasonably why you are stopping crystal growth. What about the insects, does the same sort of thing apply there or not?

P. L. Davies. Well, that is why I showed you the diagram of the fish. I did not want to have a diagram for the insects because I think it is very difficult for us to understand what they are doing. For example, the spruce budworm over winters at the tips of branches in a very exposed location and we know this is −30 °C, yet they do not freeze. Even the most concentrated solution of AFP that we can produce gives us a thermal hysteresis of about five or six degrees. That is not going to prevent those insects from freezing, so we think there must be multiple components to freeze avoidance, and the antifreeze might be useful in the spring and the autumn when there is just a few degrees of frost. It is certainly not going to protect them entirely.

D. Hall. It could actually work over a short time-scale until they can adapt to other mechanisms, as it were.

P. L. Davies. That is right, it is a possibility. There is also talk about them neutralizing ice nucleation proteins as well.

D. Hall. The interesting thing is if you look at the hysteresis, all of these things look rather similar. If you look at the overall shape, they are pretty much the same. And it seems the same thing with the insects, which is odd because one might expect a rather different mechanism and therefore a rather different curve.

P. L. Davies. I think the mechanism is basically the same, that is, binding to ice probably irreversibly. I think it is really the increased activity that has to do with the ice crystal morphology rather than the tightness of binding.

M. A. Marahiel (*Department of Chemistry, Philipps-Universität Marburg, Marburg, Germany*). What kind of concentration are we speaking about here for AFPs and α-helices? Normally, about 14 residues form a stable α-helix.

P. L. Davies. In terms of concentration, typically the flounder antifreeze is about 10 mg ml^{-1}. There are some fish that produce higher concentrations up to 20–30 mg ml^{-1}. In terms of the relationship to length, we have actually shortened the helix. The HPLC 6 that everybody seems to work on is 37 amino acids long and we have shortened it down to about 15 and it loses a lot of activity. It is still able to bind to ice and shape ice but it does not have thermal hysteresis. In the other direction, there is an isoform, AFP9, that has an additional 11 amino acid repeat and is significantly more active than the three repeat ones.

M. Smallwood (*Department of Biology, University of York, York, UK*). I wanted to ask about the relationship with recrystallization inhibition. Are the insect proteins equivalently more active in inhibiting recrystallization as they are in terms of thermal hysteresis?

P. L. Davies. I do not think we are seeing any better inhibition of ice recrystallization than with the fish proteins, so although they are hyperactive in terms of thermal hysteresis, we do not see that in inhibition of ice recrystallization.

M. Smallwood. Do you think that could relate to the way the crystal surface is bound? The insect protein binds with the *a*-axis, whereas it is a *c*-axis with the fish ones. Is there any relationship, could that impinge on their relative activity?

P. L. Davies. We have a hypothesis that the similar plant AFPs may be very good at inhibition of ice recrystallization because they have two weak ice-binding sites, and the secret of having a weak ice-binding site of course is that you do not really want to have too much depression of the freezing point before you get ice forming. Having two ice-binding sites may allow the antifreeze to bind to two grains of ice at the same time and do a better job of inhibiting recrystallization. We do not see these attributes in either the fish or the insect AFPs and this is where we really need to solve more structures for the plant antifreezes. They are really lagging behind and that is why I asked the question: do you have any information about the structure of the carrot antifreeze?

D. Smith (*Department of Chemistry, University of York, York, UK*). I have been constructing a model of the carrot protein based upon a known X-ray structure which is a leucine-rich repeat protein called the ribonuclease inhibitor. It is found in human and pigs and various creatures. The overall structure indicates that it is shaped like half a tyre and would have two flat surfaces. One at the top and one at the bottom, so maybe that is what we are looking for.

P. L. Davies. The flat surfaces are the sides of the tyre is that right?

D. Smith. Yes, the top and the bottom.

T. Haymet (*Department of Chemistry, University of Houston, Houston, TX, USA*). My question concerns your comparison of the 20 mM of the insect antifreeze and the 400 mM of fish antifreeze. We find that, for *Tenebrio molitor* protein, that time or cooling rate is a very important factor. A solution of that concentration left at just a few tenths of degree of supercooling would, after many hours grow ice crystals, whereas your picture implied that there was no time-scale, just temperature.

P. L. Davies. We have a certain regime for measuring thermal hysteresis using a nanolitre osmometer which will decrease 10 milliosmoles every 15 s. We keep to that rate and that is how we put everything on an equal footing. However, I will say that we have at times using Mike Kuiper's capillary cooling apparatus introduced an ice crystal in there and monitored it over a period of days, and we see absolutely no growth. It is really quite remarkable; you go back and take a picture a day later, two days later and that ice crystal still has not changed at all and it will be a certain temperature below the true freezing point.

T. Haymet. In our hands, we find that time is a very important factor and that these extreme claims of supercooling for insect antifreeze really only occur on the short time-scale so that, at 20 mM, I would argue that these have a few tenths of a degree supercooling.

C. Knight (*National Center for Atmospheric Research, Boulder, CO, USA*). I must say, I find your interpretation of the ice-binding side of the antifreezes very convincing.

But I wonder if you have thought at all about how to fit the glycopeptides into this picture. They seem to me to be very difficult to handle.

P. L. Davies. Yes, it is ironic that they were the first to be characterized and they are probably the last ones to have their structure and function worked out. I know it was proposed that the sugars might be binding to ice and the idea again was that, in the days of the hydrogen-bonding hypothesis, that you have lots of hydroxyls on the sugars and they might bind to ice. I suspect that in fact the other side of the glycoprotein might be the key to binding very much in the way that the surface of the sculpin and flounder helices are binding to ice. My prediction would be that methyl groups are actually binding to the ice in conjunction with a little part of the sugar. Now people have been able to synthezise the antifreeze glycoprotein so I am hoping that somebody will come up with some structure–function studies where they are changing some of those alanines to leucine and maybe modifying the sugars. I think it is within the grasp of being demonstrated, which is the ice-binding surface.

GLOSSARY

AFP: antifreeze protein
CfAFP: *Choristoneura fumiferana* AFP
TmAFP: *Tenebrio molitor* AFP